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Synergistic Triggering of Superoxide Flashes by Mitochondrial Ca^{2+} Uniport and Basal Reactive Oxygen Species Elevation*[S]

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Tingting Hou[‡1], Xing Zhang[§1], Jiejia Xu[‡], Chongshu Jian[‡], Zhanglong Huang[‡], Tao Ye[¶], Keping Hu[‖], Ming Zheng[‡], Feng Gao[§], Xianhua Wang[‡2], and Heping Cheng[‡]

From the [‡]State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Molecular Medicine, Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China, the [§]Department of Physiology, Fourth Military Medical University, Xi'an 710023, China, the [¶]Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University, Hong Kong, China, and the [‖]The Research Center for Pharmacology and Toxicology, the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100094, China

Background: Superoxide flashes are newly discovered elemental mitochondrial ROS signaling events and reflect transient openings of mPTP.
Results: Mitochondrial Ca^{2+} uniport and basal ROS elevation synergistically trigger superoxide flashes.
Conclusion: Superoxide flashes are regulated by physiological levels of Ca^{2+} and ROS.
Significance: Demonstrating Ca^{2+}- and ROS-induced flash activation sheds new light on physiological regulation and possible roles of mPTP.

Mitochondrial superoxide flashes reflect a quantal, bursting mode of reactive oxygen species (ROS) production that arises from stochastic, transient opening of the mitochondrial permeability transition pore (mPTP) in many types of cells and in living animals. However, the regulatory mechanisms and the exact nature of the flash-coupled mPTP remain poorly understood. Here we demonstrate a profound synergistic effect between mitochondrial Ca^{2+} uniport and elevated basal ROS production in triggering superoxide flashes in intact cells. Hyperosmotic stress potently augmented the flash activity while simultaneously elevating mitochondrial Ca^{2+} and ROS. Blocking mitochondrial Ca^{2+} transport by knockdown of MICU1 or MCU, newly identified components of the mitochondrial Ca^{2+} uniporter, or scavenging mitochondrial basal ROS markedly diminished the flash response. More importantly, whereas elevating Ca^{2+} or ROS production alone was inefficacious in triggering the flashes, concurrent physiological Ca^{2+} and ROS elevation served as the most powerful flash activator, increasing the flash incidence by an order of magnitude. Functionally, superoxide flashes in response to hyperosmotic stress participated in the activation of JNK and p38. Thus, physiological levels of mitochondrial Ca^{2+} and ROS synergistically regulate stochastic mPTP opening and quantal ROS production in intact cells, marking the flash as a coincidence detector of mitochondrial Ca^{2+} and ROS signals.

An organelle of endosymbiotic origin, the mitochondrion displays amazing functional versatility in eukaryotic cells. Not only does it act as the so-called "powerhouse" central to bioenergetics, but it also plays pivotal roles in Ca^{2+} handling, redox homeostasis, signal transduction, and cell fate regulation. The inner membrane of the mitochondrion comprises the distinctive lipid cardiolipin and exhibits a low permeability that safeguards steep transmembrane potentials ($\Delta\Psi_m$ is approximately -180 mV), preserves proton gradients (ΔpH $\sim$0.8), and partitions a protein-crowded, highly oncotic matrix from its immediate cytosolic environment. Nevertheless, a phenomenon called membrane permeability transition (MPT)[3] has been reported in isolated mitochondria (1–3): a sudden loss of the inner membrane impermeability that allows the free passage of ions, water, and small solutes with molecular masses up to 1,500 Da. Further studies have revealed two gating modes of the underlying mitochondrial permeability transition pore (mPTP): irreversible, full conductance opening for the permanent MPT (pMPT) and reversible opening with variable smaller conductance for the transient MPT (tMPT) (4–8). Functionally, the irreversible, all or none pMPT is associated with the release of proapoptotic factors from the mitochondria on the verge of cell death (9–11), whereas the tMPT, which may occur stochastically under physiological or mild to moderate stress conditions (12), participates in biological processes including prenatal cardiac development (13) and cell-protective (*e.g.*, discharge of mitochondrial Ca^{2+} overload) (14) and preconditioning signal transduction (*e.g.*, ROS-dependent activation of cytosolic protein kinase C) (15, 16).

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[S] This article contains supplemental Figs. S1–S4 and Movies S1–S3.
[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed: State Key Laboratory of Biomembrane and Membrane Biotechnology, Inst. of Molecular Medicine, Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China. Tel.: 86-10-62754605; Fax: 86-10-62765957; E-mail: xianhua@pku.edu.cn.

[3] The abbreviations used are: MPT, membrane permeability transition; pMPT, permanent MPT; tMPT, transient MPT; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; CsA, cyclosporine A; CIFA, Ca^{2+}-induced flash activation; RIFA, ROS-induced flash activation; CRIFA, Ca^{2+}- and ROS-induced flash activation.

With the aid of a novel, reversible superoxide biosensor, mt-cpYFP, we recently demonstrated that tMPT is tightly coupled to quantal bursting ROS production, giving rise to discrete, single-mitochondrion events called "superoxide flashes" (17). As a fundamental mitochondrial phenomenon, superoxide flashes occur in many types of cells (17–20), in the *ex vivo* beating heart (17), and even in the skeletal muscle and peripheral nerve of living animals (21, 22) and are stereotypical in terms of amplitude and kinetics. Unlike basal mitochondrial ROS production caused by electron leakage from the electron transport chain, superoxide flash ignition is tightly coupled to the tMPT, evidenced by sudden and reversible dissipation of $\Delta\Psi_m$, irreversible partial loss of small solutes (molecular weight = ~752–980) from the matrix, and transient mitochondrial swelling (17, 18, 20). The flash-linked tMPT is sensitive to cyclosporine A (CsA) (17, 18, 20) (see also Refs. 19 and 22 for skeletal muscle as a notable exception), and cyclophilin D acts as an important but dispensable regulator (17, 18, 20), as is the case with the pMPT (23–26).

Furthermore, tMPT-coupled superoxide flashes are thought to be local, elemental ROS signaling events. They make only a miniscule contribution to global ROS in setting the overall redox status of the cell (18), but their incidence is regulated by an array of factors that converge on the mPTP and the electron transport chain complexes (20). In particular, superoxide flashes decode metabolic status in a frequency-dependent manner in living mice (21), respond to reoxygenation stress after hypoxia or complete anoxia (17, 27), and constitute an early signal of oxidative stress-induced apoptosis, independently of mitochondrial release of proapoptotic factors such as cytochrome *c* (18).

An important unanswered question is whether and how the two types of MPT, the flash-coupled tMPT and the irreversible pMPT, are differentially regulated. Answering this question would provide new understanding of another still elusive issue: whether the two underlying mPTPs are of the same or distinct molecular identity. In the present study, we used superoxide flashes as a means to record tMPT at the single mitochondrion level in intact cells and to determine whether and how superoxide flashes are regulated by mitochondrial Ca^{2+} and ROS, two well characterized regulators of the pMPT. Our results showed a profound synergism between the physiological levels of mitochondrial Ca^{2+} and elevated ROS production in triggering superoxide flashes, revealing similarities and disparities between the two types of mPTPs in terms of their Ca^{2+} and ROS regulation.

EXPERIMENTAL PROCEDURES

Reagents—Cyclosporine A, bongkrekic acid, carboxyatractyloside, D-glucose, D-mannitol, EGTA, histamine, ionomycin, menadione, and mitoTEMPO were from Sigma. MitoSOX was from Molecular Probes (Eugene, OR), and Lipofectamine RNAiMax was from Invitrogen. SS31 (D-Arg-Dmt-Lys-Phe-NH_2) was synthesized as described (28). Phospho-JNK and total JNK antibodies were from Cell Signaling Technology, and phospho-p38 and total p38 antibodies were from Santa Cruz.

Cell Culture and RNA Interference Assay—Stable expression of mitochondria-targeted cpYFP (mt-cpYFP) in HeLa cells was established as previously described (18). The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin and streptomycin (Invitrogen) at 37 °C under 5% CO_2. The double-stranded RNA sequences for knockdown of human MICU1 and MCU and their negative control are shown in Table 1.

TABLE 1
RNA sequences for knockdown

Name	RNA sequences
MICU1-a sense	GCAGCUCAAGAAGCACUUCAA dTdT
MICU1-a antisense	UUGAAGUGCUUCUUGAGCUGC dTdT
MICU1-b sense	GCAAUGGCGAACUGAGCAAUA dTdT
MICU1-b antisense	UAUUGCUCAGUUCGCCAUUGC dTdT
MCU-a sense	CCAGCAACUAUACACCACACU dTdT
MCU-a antisense	AGUGUGGUGUAUAGUUGCUGG dTdT
MCU-b sense	GCAAGGAGUUUCUUUCUCUUU dTdT
MCU-b antisense	AAAGAGAAAGAAACUCCUUGC dTdT
NC sense	UUCUCCGAACGUGUCACGU dTdT
NC antisense	ACGUGACACGUUCGGAGAA dTdT

For transfection, 100 nM siRNA was transiently transfected into the mt-cpYFP-expressing HeLa cells with Lipofectamine RNAiMax according to the manufacturer's instructions. Knockdown efficiency was tested by Western blot.

Ca^{2+} Measurement—Fluo-4 AM was used to measure the cytosolic Ca^{2+} signal, and Rhod-2 AM was used to measure the mitochondrial Ca^{2+} signal according to the manufacturer's instructions. Briefly, HeLa cells were incubated in the presence of Fluo-4 AM (5 μM) or Rhod-2 AM (5 μM) at 37 °C for 15–30 min. After washing with Tyrode's solution consisting of 137 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, and 20 mM Hepes (pH 7.35, adjusted with NaOH), Fluo-4 and Rhod-2 fluorescence was measured with a Zeiss LSM 710 confocal microscope.

Measurement of Mitochondrial Basal ROS—Mitochondrial basal ROS was measured with mitoSOX according to the manufacturer's instructions. Briefly, HeLa cells were incubated in the presence of mitoSOX at 37 °C for 20 min and then washed with Tyrode's solution. Basal ROS was indexed by the slope of the time-dependent rise in mitoSOX fluorescence.

Confocal Imaging—Imaging measurement of mt-cpYFP, tetramethyl rhodamine methyl ester, Fluo-4, Rhod-2, and mitoSOX signals was carried out on a Zeiss LSM 710 confocal microscope equipped with a 40×, 1.3 NA oil immersion objective, when cells were bathed in Tyrode's solution. When acquiring the mt-cpYFP signal alone, the images were acquired by exciting alternately at 488 and 405 nm and collecting the emission at >505 nm. To obtain mt-cpYFP and tetramethyl rhodamine methyl ester signals simultaneously, images were taken by exciting sequentially at 488, 405, and 543 nm and collecting the emission at 505–530, 505–530, and >560 nm, respectively. For Fluo-4 fluorescence, the images were acquired by exciting at 488 nm and collecting the emission at 505–530 nm. For Rhod-2 fluorescence, the images were acquired by exciting at 543 nm and collecting the emission at >560 nm. For mitoSOX fluorescence, the images were acquired by exciting at 514 nm and collecting the emission at 580–740 nm. In a given cell, usually 100 frames of 512 × 512 (xy) pixels were collected at 1 s/frame in bidirectional scanning mode. The axial resolution was set to 1.0 μm, and the size of the imaging region was 212.13 × 212.13

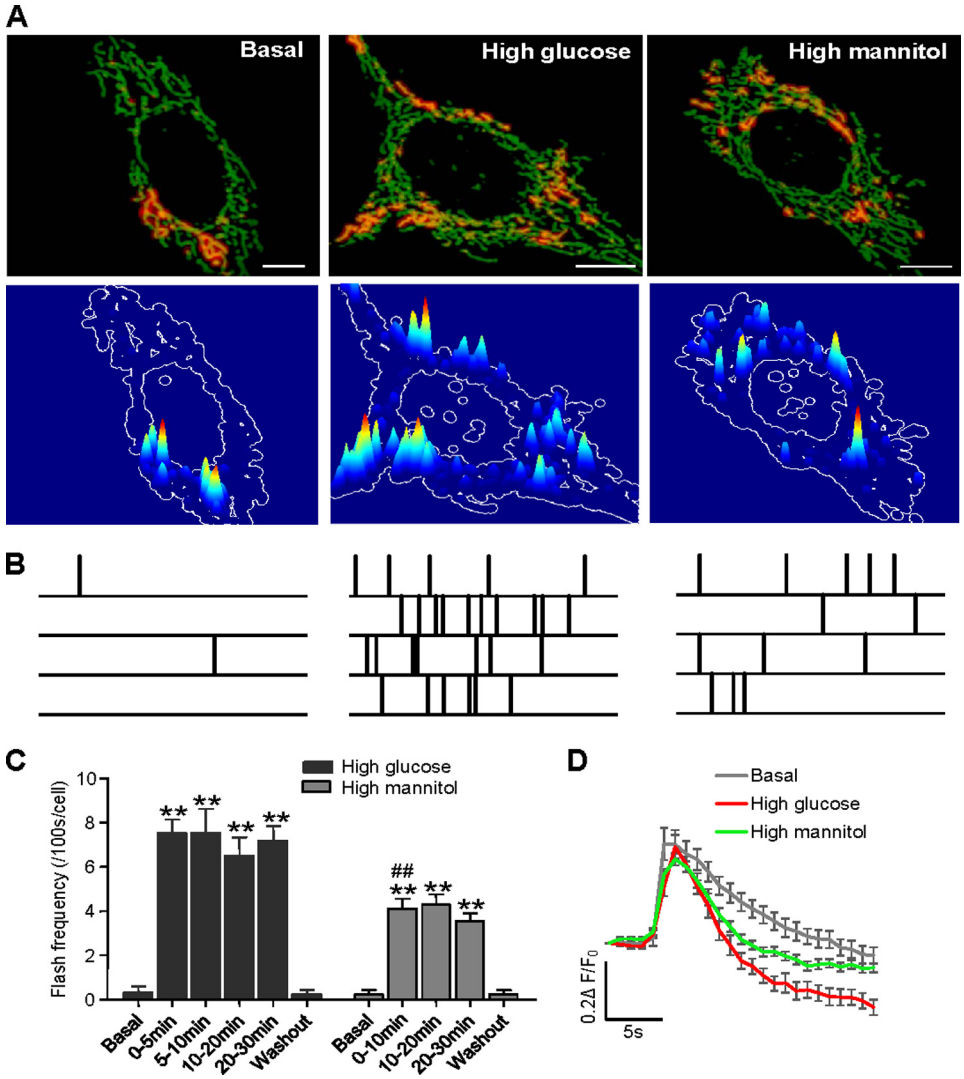

FIGURE 1. **Hyperactive superoxide flashes induced by hyperosmotic stress.** *A*, representative deviation maps (*upper row*) and surface plots (*lower row*) of superoxide flashes registered in HeLa cells expressing mt-cpYFP. The data were extracted from time lapse image stacks (100 frames, 1 s/frame) in basal, high glucose (270 mM D-glucose in Tyrode's solution), and high mannitol (270 mM D-mannitol in Tyrode's solution). *Scale bars*, 10 μm. *B*, representative diaries of superoxide flash incidence. Each *vertical tick* denotes a flash event. *C*, time course of the flash response to high glucose or mannitol. The data are expressed as the means ± S.E. ($n \geq 15$ cells/group). **, $p < 0.01$ high glucose or mannitol *versus* basal; ##, $p < 0.01$ high mannitol *versus* high glucose. *D*, time courses of averaged superoxide flashes in basal (*gray*), high glucose (*red*), and high mannitol (*green*). The data are expressed as the means ± S.E. ($n = 15$–46 flashes for each trace).

μm. Image acquisition was usually completed within 40 min after different treatments. All of the experiments were performed at room temperature (22–26 °C) unless specified otherwise.

Imaging Data Analysis—The input data set was a time lapse LSM file generated from the Zeiss LSM 710. Confocal images were analyzed using custom-developed programs written in interactive data language (IDL Research Systems).

Statistics—The data are expressed as the means ± S.E., and Student's *t* test was applied to determine the statistical significance. $p < 0.05$ was considered statistically significant.

RESULTS

Superoxide Flashes Induced by Hyperosmotic Stress—In HeLa cells stably expressing the mitochondrial superoxide biosensor mt-cpYFP, time lapse confocal imaging visualized a flurry of superoxide flash activity that occurred immediately upon challenge with high glucose (270 mM in Tyrode's solution). Individual flashes were identified as sudden, discrete ROS bursts that were ignited randomly over space and time (Figs. 1, *A*–*C*, and supplemental Movies S1 and S2). On average, the flash incidence reached 5.72 ± 0.52 events/100 s/cell as seen in 1-μm-thick confocal optical sections; this was 19-fold greater than that in control cells. Likewise, a 12-fold increase of superoxide flash incidence (2.65 ± 0.29 events/100 s/cell) was registered in the presence of metabolically inactive hyperosmotic mannitol (270 mM in Tyrode's solution) (Fig. 1, *A*–*C*, and supplemental Movie S3). Individual flashes were sharply compartmentalized to punctiform or filamentous mitochondria or arose from synchronous activation of convoluted mitochondrial networks of variable shape and size (Fig. 1 and supplemental Movies S1–S3).

The hyperactivity of superoxide flashes developed within the first a few minutes of the hyperosmotic challenge and persisted unabatedly over the entire period of observation of 30 min but completely returned to its basal level after switching back to

normal Tyrode's solution (Fig. 1*C*). The reversibility of the flash response, in conjunction with the lack of major changes in mitochondrial network morphology (Fig. 1, *A* and *C*), attests to the functional integrity of mitochondria under these experimental conditions. Thus, hyperactivity of superoxide flashes constitutes a prominent, heretofore unappreciated mitochondrial response to hyperosmotic stress.

Despite the large increase in flash incidence, there was little change in average flash amplitude (0.32 $\Delta F/F_0$ of mt-cpYFP fluorescence) and rise time (~1.5 s). The decay of the flashes, however, was accelerated from ~8.2 s in control to ~4.0 s in the presence of high osmolytes (Fig. 1*D* and supplemental Fig. S1*A*). Hyperactive superoxide flashes were sensitive to CsA (2 μM), which inhibits the mPTP regulator cyclophilin D (supplemental Fig. S1*C*). Bongkrekic acid (20 μM) and carboxyatractyloside (10 μM), which cause a conformation-dependent inhibition and activation of mPTP through binding adenine nucleotide translocator, decreased and increased the incidence of superoxide flashes, respectively (supplemental Fig. S1*C*). Furthermore, the onset of the flash coincided with a transient loss of $\Delta\Psi_m$ as indexed by tetramethyl rhodamine methyl ester fluorescence (supplemental Fig. S1*B*), supporting an involvement of transient mPTP opening in the ignition of superoxide flashes in hyperosmotic stress.

Mitochondrial Ca^{2+} Uniport Was Required for Hyperosmotic Stimulation of Superoxide Flashes—The hyperactivity of superoxide flashes in osmotic stress affords a unique experimental setting to investigate the regulatory mechanisms underlying the genesis of superoxide flashes. Given that the irreversible mPTP activity is critically sensitive to mitochondrial Ca^{2+} overload and oxidative stress, we sought to determine the possible involvement of intracellular Ca^{2+} and ROS signals in triggering transient mPTP opening (using the flash as an optical readout).

Application of high glucose immediately elicited a cytosolic Ca^{2+} transient that displayed a 1.58 ± 0.09-fold peak increase of Fluo-4 fluorescence and gradually declined toward an elevated steady level of 0.51 ± 0.03 ($n = 90$ cells, $\Delta F/F_0$ at 10 min). A similar, but smaller, Ca^{2+} response was elicited by high mannitol, with peak and steady-state levels of 0.80 ± 0.03 and 0.43 ± 0.03 ($n = 51$ cells, at 10 min), respectively (Fig. 2*A*). These results are in agreement with previous reports that hyperosmotic challenge stimulates both Ca^{2+} entry and intracellular store Ca^{2+} release in canine kidney cells, rat terminal inner medullary collecting ducts, and human keratinocytes (29). Removal of extracellular Ca^{2+} markedly lowered the basal Ca^{2+} level and limited the maximal attainable Ca^{2+} to a level merely comparable with or even smaller than the normal resting Ca^{2+} concentration (Fig. 2*A*). Concomitantly, the flash response to hyperosmotic glucose was suppressed by 81.1% and to mannitol by 43.4% in the absence of extracellular Ca^{2+} (Fig. 2*B*). This result provides the first evidence suggesting that physiologically elevated intracellular Ca^{2+} participates in the regulation of superoxide flash activity (see "Discussion" for a previous report in cardiomyocytes (17)). By contrast, pMPT is known to operate only at supraphysiological levels of high Ca^{2+} (23–26, 30).

In HeLa cells with mitochondrial loading of the Ca^{2+} indicator Rhod-2, hyperosmotic challenge induced robust mitochondrial Ca^{2+} transients, with temporal features similar to those of the aforementioned cytosolic Ca^{2+} transients (Fig. 2*C*). With exceptions, the mitochondrial Ca^{2+} uniport provides the major route to couple cytosolic Ca^{2+} transients with mitochondrial matrix Ca^{2+} elevation; the latter directly regulates diverse physiological and pathophysiological mitochondrial processes including the Krebs cycle and mPTP activity (31–34). Recently, MICU1 and MCU have been identified as the Ca^{2+} sensor (35) and putative channel pore elements of the mitochondrial Ca^{2+} uniporter (36, 37). To disrupt the mitochondrial Ca^{2+} uniporter, we performed siRNA knockdown of MICU1 or MCU with two independent double-stranded small RNAs for either gene (supplemental Fig. S2). As a result, mitochondrial Ca^{2+} elevation responding to high glucose or mannitol was decreased by 51.8–77.1% with either MICU1 or MCU knockdown (Fig. 2, *C* and *D*). The decrease in mitochondrial Ca^{2+} response was accompanied by a 51.6–65.9% decrease in the flash response to high glucose (33.1–37.5% decrease with mannitol) (Fig. 2*E*). These data indicate that the mitochondrial Ca^{2+} uniport is causatively linked to hyperactivity of superoxide flashes in hyperosmotic stress. It is noteworthy that the mitochondrial Ca^{2+} elevation associated with hyperosmotic stress was only moderate (Rhod-2 $\Delta F/F_0$ <3.0), marking an important difference between mitochondrial Ca^{2+} triggering of stochastic mPTP openings in hyperosmotic stress and that of irreversible mPTP openings induced by injurious matrix Ca^{2+} overload.

Increasing Mitochondrial Ca^{2+} Alone Failed to Activate Superoxide Flashes—Next, we sought to determine whether mitochondrial Ca^{2+} transients elicited by means other than hyperosmotic stress are equally potent in activating superoxide flash production. Application of histamine (100 μM) elicited robust cytosolic and mitochondrial Ca^{2+} transients (Fig. 3*A*), by stimulating extracellular Ca^{2+} entry and mobilizing store Ca^{2+} from the endoplasmic reticulum via receptor-mediated signaling pathways (38, 39). The peak amplitudes of the cytosolic and mitochondrial Ca^{2+} transients were 4.03 ± 0.03 ($\Delta F/F_0$ of Fluo-4 fluorescence, $n = 100$ cells) and 2.94 ± 0.09 (Rhod-2 $\Delta F/F_0$, $n = 100$ cells), respectively. Surprisingly, we found that histamine failed to stimulate any detectable increase in superoxide flash activity, even within the first 5 min when the mitochondrial Ca^{2+} level was comparable with that stimulated by high glucose or even greater than that in high mannitol (Fig. 3*C*). Because histamine-elicited Ca^{2+} transients exhibited no appreciable steady-state component, we applied ionomycin (2 μM), a Ca^{2+} ionophore, to activate cytosolic and mitochondrial Ca^{2+} elevations (Fluo-4 $\Delta F/F_0$ 5.70 ± 0.14, $n = 90$ cells; and Rhod-2 $\Delta F/F_0$ 2.63 ± 0.18, $n = 90$ cells) with a sizable steady-state mitochondrial component (Rhod-2 $\Delta F/F_0$ 0.27 ± 0.03) (Fig. 3*B*). Still, neither transient nor steady-state mitochondrial Ca^{2+} elevation augmented the flash incidence (Fig. 3*C*). Thus, in contrast to the strong Ca^{2+} dependence of hyperosmotic stress-stimulated superoxide flashes, increasing mitochondrial Ca^{2+} by histamine or a low concentration of ionomycin is insufficient to activate flash activity.

ROS-induced Activation of Superoxide Flash—The paradoxical results depicted above suggest that mitochondrial Ca^{2+} regulation of the flash activity is contextual, and additional co-

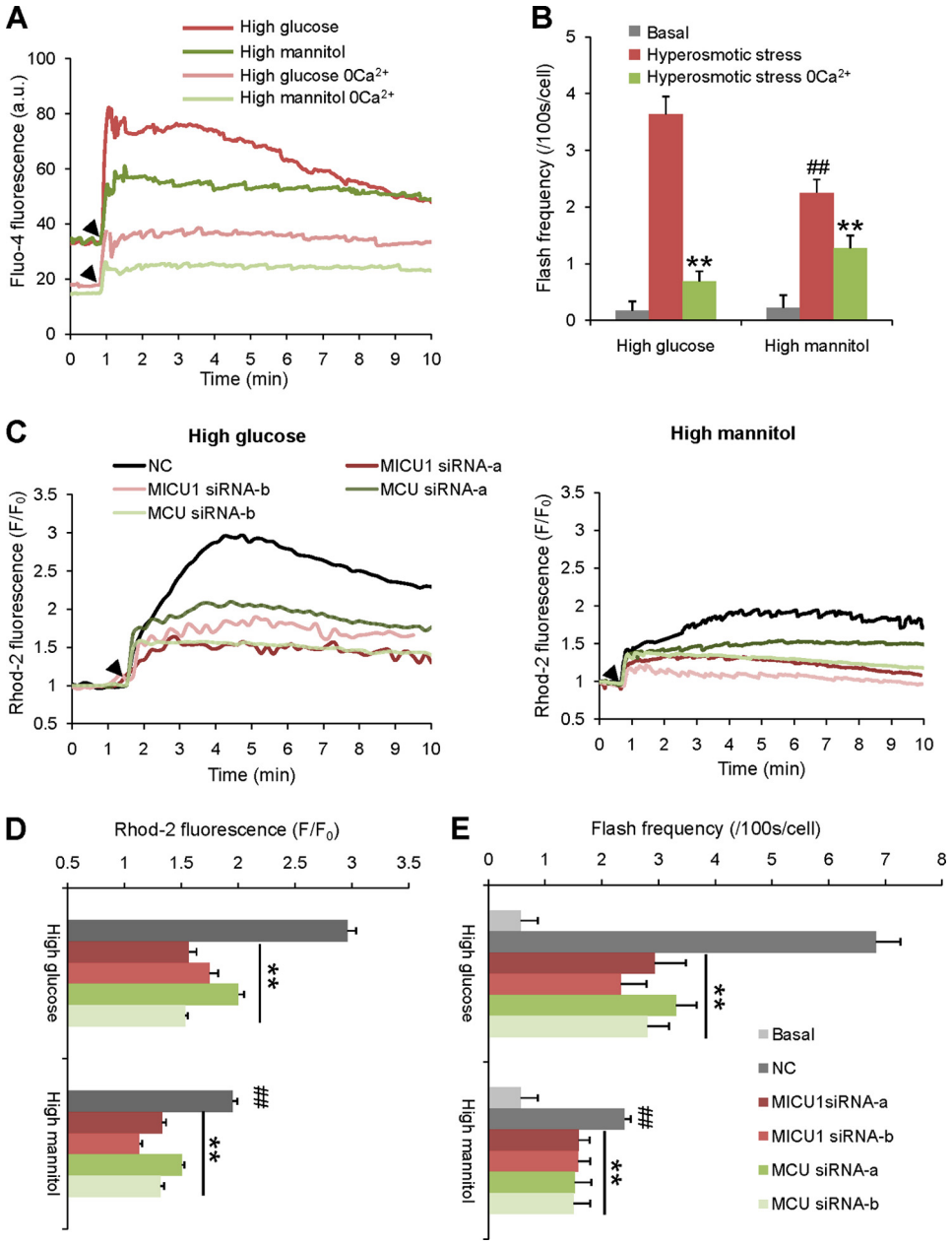

FIGURE 2. **Mitochondrial Ca^{2+} uniport is required for hyperosmotic stimulation of superoxide flashes.** *A*, cytosolic Ca^{2+} (measured with Fluo-4) responses to high glucose and high mannitol in the presence or absence of extracellular Ca^{2+} ($n = 50–90$ cells for each trace). The *error bars* are omitted for clarity. *Arrowheads* mark the time of hyperosmotic stimulation. *0Ca²⁺*: 5 mM EGTA in 0 Ca^{2+} Tyrode's solution; *a.u.*, arbitrary units. *B*, removal of extracellular Ca^{2+} decreased hyperosmosis-associated flash activity. The data are expressed as the means ± S.E. ($n \geq 16$ cells/group). **, $p < 0.01$ *versus* normal Ca^{2+}; ##, $p < 0.01$ *versus* high glucose. *C*, effect of MICU1 or MCU knockdown on mitochondrial Ca^{2+} (measured with Rhod-2) responses to high glucose (*left panel*) and high mannitol (*right panel*). *NC*, siRNA negative control; *MICU1 siRNA-a* and *MICU1 siRNA-b*, two siRNAs for knocking down MICU1; *MCU siRNA-a* and *MCU siRNA-b*, two siRNAs for knocking down MCU; F_0, basal Rhod-2 fluorescence; F/F_0, normalized fluorescence signal. *Arrowheads* indicate the time of hyperosmotic stimulation. *D*, statistics of Rhod-2 amplitude as shown in *C*. The data are expressed as the means ± S.E. ($n \geq 50$ cells/group). **, $p < 0.01$ *versus* negative control; ##, $p < 0.01$ *versus* high glucose. *E*, effect of MICU1 or MCU knockdown on superoxide flash activity induced by hyperosmotic stress. The data are expressed as the means ± S.E. ($n \geq 20$ cells/group). **, $p < 0.01$ *versus* negative control; ##, $p < 0.01$ *versus* high glucose.

factor must be involved, which greatly potentiates mitochondrial Ca^{2+} for the full-fledged flash response in hyperosmotic stress; its absence in circumstances such as histamine or ionomycin stimulation may account for the inability or poor efficiency of mitochondrial Ca^{2+} in activating superoxide flashes.

As an initial clue, we noticed that hyperosmotic stress reversibly increased the background mt-cpYFP fluorescence (supplemental Fig. S3), consistent with previous studies that hyperosmotic stress elevates cytosolic ROS (29). Independent measurement using the slope of rise of the fluorescence of mitoSOX, which reports the mitochondrial superoxide level by an irreversible oxidation reaction (40, 41), revealed enhanced basal ROS production in the presence of high glucose or high mannitol. On average, the slope of the mitoSOX fluorescence rise was increased by 2.20 ± 0.3-fold in high glucose (Fig. 4, *A* and *B*) or 2.16 ± 0.1-fold in high mannitol (Fig. 4*B*). To test the hypothesis that elevated mitochondrial ROS also contributes to superoxide flash hyperactivity in hyperosmotic stress, we pre-

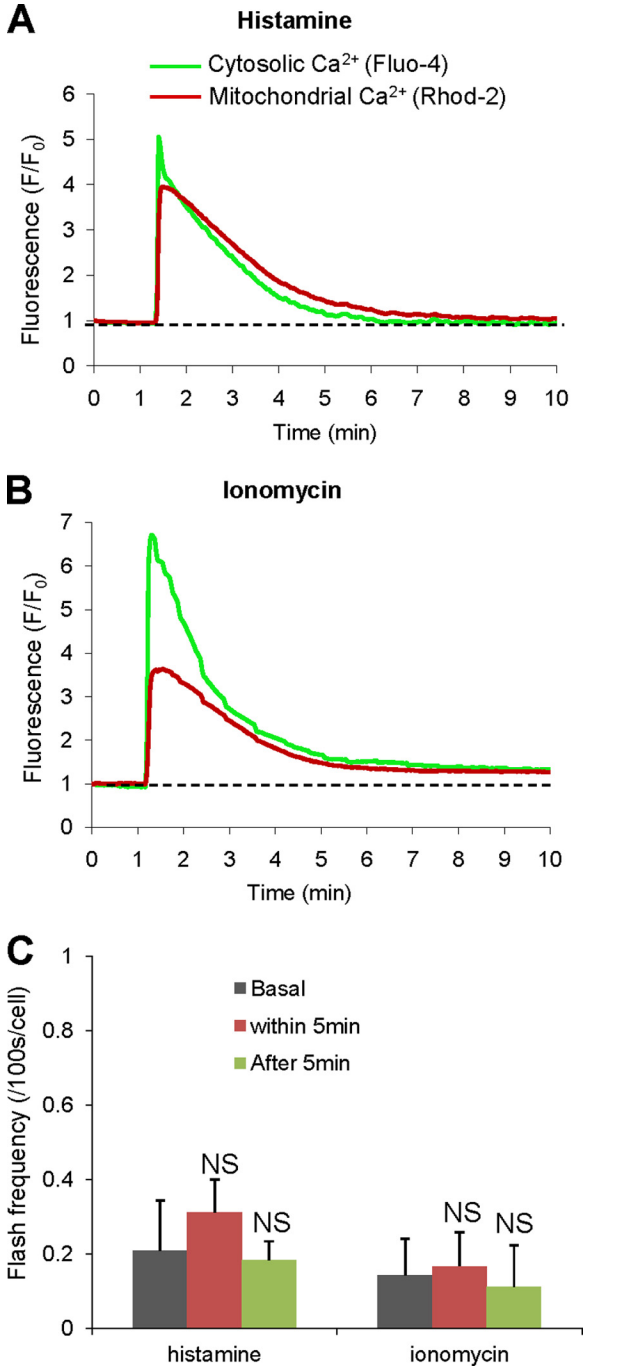

FIGURE 3. **Increasing mitochondrial Ca^{2+} alone is insufficient to augment superoxide flash activity.** *A* and *B*, cytosolic and mitochondrial Ca^{2+} transients stimulated by 100 μM histamine (*A*) or 2 μM ionomycin (*B*) (n = 70–100 cells for each trace). The *error bars* are omitted for clarity. *C*, histamine or ionomycin treatment was unable to alter superoxide flash activity. The data are expressed as the means ± S.E. ($n \geq 10$ cells/group). *NS*, no significant difference.

treated cells with a mitochondria-targeted antioxidant, mito-TEMPO (500 nM) (42), or the tetrapeptide SS31 (100 μM) (28, 43). Our results showed that both treatments effectively prevented hyperosmotic stress-associated mitochondrial basal ROS production (Fig. 4, *A* and *B*). Concomitantly, the flash response to high glucose was attenuated by 37–51%, and that to high mannitol was attenuated by 31–44% (Fig. 4*C*). Thus, the hyperactivity of superoxide flashes in hyperosmotic stress depends also on mitochondrial basal ROS production.

Further, to assess the efficiency of mitochondrial basal ROS as the flash activator independent of mitochondrial Ca^{2+} elevation, we elevated mitochondrial basal ROS production using different concentrations of menadione, a quinone that undergoes one-electron reduction to produce superoxide directly and depletes intracellular thiols to indirectly stimulate mitochondrial superoxide production (44, 45). As expected, menadione increased the slope of rise of the mitoSOX fluorescence in a dose-dependent manner (Fig. 4, *D* and *E*). At 25 μM, menadione induced a 2.08 ± 0.12-fold increase in mitochondrial basal ROS production, which was comparable with that in hyperosmotic stress. However, it failed to exert any significant effect on superoxide flash activity. At concentrations of 50 μM or higher, menadione dose-dependently increased the superoxide flash activity (Fig. 4*F*), to a level comparable with that induced by high glucose at the highest concentration tested (200 μM), when the basal ROS production rate was 3.5-fold greater than that in hyperosmotic stress (Fig. 4, *E* and *F*). These data indicate that mitochondrial basal ROS activates superoxide flash production but does so inefficaciously compared with ROS in hyperosmotic stress.

Synergistic Effect of Mitochondrial Ca^{2+} and ROS in Triggering Superoxide Flashes—The data thus far suggested that both mitochondrial Ca^{2+} and basal ROS elevation are required for full expression of the superoxide flash response to hyperosmotic stress, whereas mitochondrial Ca^{2+} or ROS acting alone is ineffective or inefficient as the flash activator. To directly test this hypothesis, we simultaneously applied subthreshold concentrations of menadione (25 μM) and ionomycin (2 μM). Combined, they raised the rate of superoxide flash occurrence to 3.5 ± 0.54 events/100 s/cell in a CsA-sensitive manner (Fig. 5*A*). Importantly, this was completely abolished either by the removal of extracellular Ca^{2+} (negating mitochondrial Ca^{2+} elevation) or the presence of the tetrapeptide antioxidant SS31 (negating mitochondrial basal ROS elevation) (Fig. 5*A*). Time course analysis further revealed that the response to combined mitochondrial Ca^{2+} and ROS elevation was fully developed in the first 100 s of treatment and persisted at a high steady level for the entire period of observation. This supralinear response corroborates a synergistic interplay between combined Ca^{2+} and ROS signals.

Qualitatively similar results were obtained when subthreshold menadione was combined with histamine stimulation. However, the flash response was rather transient, with only a marginal steady-state component. Compared with the synergistic interaction between ionomycin and menadione, the lack of sustained response to combined histamine and menadione can be explained by the lack of a steady-state Ca^{2+} response: sustained cytosolic and mitochondrial Ca^{2+} elevation was seen only in the presence of ionomycin ($\Delta F/F_0$ 0.33 ± 0.03 in Fluo-4 and 0.27 ± 0.03 in Rhod-2, n = 90 cells) but not histamine ($\Delta F/F_0$ 0.04 ± 0.03 in Fluo-4 and 0.04 ± 0.02 in Rhod-2, n = 100 cells; Figs. 3*A* and 5, *B* and *C*). This result also attests the power of concurrent mitochondrial Ca^{2+} and ROS signals to sustain superoxide flash hyperactivity.

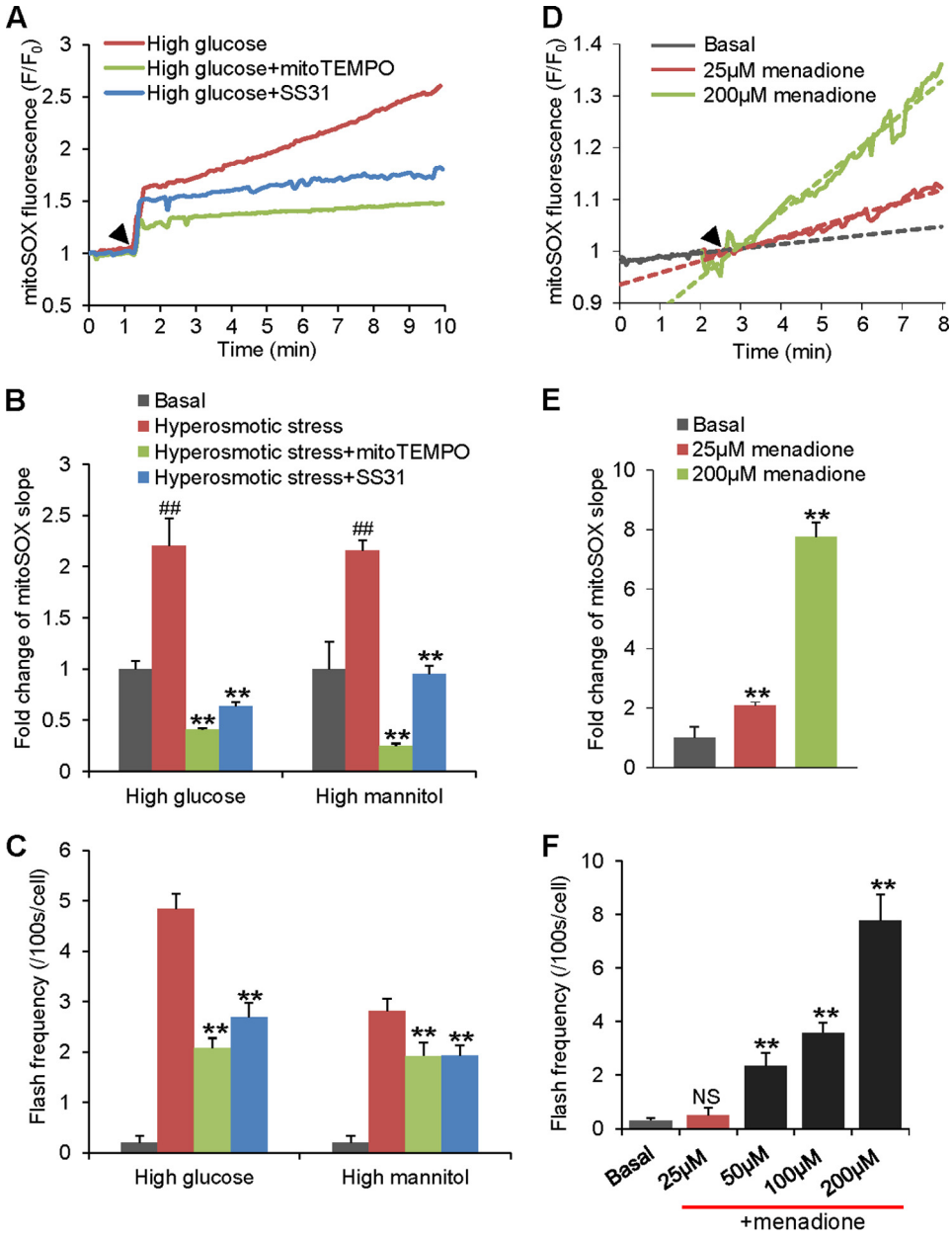

FIGURE 4. **Mitochondrial ROS elevation augments superoxide flash activity.** *A*, hyperosmotic stress-induced mitochondrial ROS production (measured with mitoSOX) in the absence and presence of the mitochondria-targeted ROS scavengers, mitoTEMPO (500 nM) or SS31 (100 μM). *Arrowhead* indicates the time of hyperosmotic stimulation ($n \geq 50$ cells for each trace). *B*, statistics. The mitochondrial ROS level was indexed by the slope of the mitoSOX signal. *Basal*, before hyperosmotic stimulation; *hyperosmotic stress*, high glucose or high mannitol stimulation; *mitoTEMPO*, pretreatment with 500 nM mitoTEMPO; *SS31*, pretreatment with 100 μM SS31. The data are expressed as the means ± S.E. ($n \geq 30$ cells/group). ##, $p < 0.01$ *versus* basal; **, $p < 0.01$ SS31 or mitoTEMPO treatment *versus* untreated group. *C*, effect of mitochondrial ROS scavengers on superoxide flash activity induced by hyperosmotic stress. The data are expressed as the means ± S.E. ($n = 11–66$ cells/group). **, $p < 0.01$ SS31 or mitoTEMPO treatment *versus* untreated group. *D*, menadione treatment increased mitochondrial basal ROS production. An *arrowhead* indicates the time of menadione addition. *Dashed lines* represent linear fitting of the averaged mitoSOX traces before (*gray*) and after addition of 25 μM (*red*) or 200 μM menadione (*green*) ($n = 11–34$ cells). *E*, statistics of *D*. The data are the means ± S.E. ($n = 11–34$ cells). **, $p < 0.01$ *versus* basal group. *F*, menadione treatment increased superoxide flash frequency in a dose-dependent manner. The data are expressed as the means ± S.E. ($n = 12–43$ cells). **, $p < 0.01$ *versus* group without menadione treatment. *NS*, no significant difference.

For synergistic effect of mitochondrial ROS and Ca^{2+} on superoxide flashes induced by hyperosmotic stress, we treated MCU-down-regulated cells with SS31. Superoxide flashes induced by hyperosmotic glucose were further depressed compared with either MCU knockdown or SS31 treatment alone, but it failed to completely abrogate the increase in flash (supplemental Fig. S4). These data suggest that signals other than mitochondrial Ca^{2+} and basal ROS also contribute to regulate superoxide flashes and provide another evidence for the synergistic effect of mitochondrial Ca^{2+} uniport and basal ROS elevation on superoxide flashes (*i.e.*, the blocking effect of two simultaneous treatments was smaller than the sum of each separate treatment).

Mitochondrial Superoxide Flashes Induced by Hyperosmotic Stress Participate in Activating JNK and P38—Hyperosmotic stress activates MAPK, which is essential for the induction of adaptive responses required for cell survival (29, 46, 47). Importantly, we found that inhibition of mitochondrial superoxide

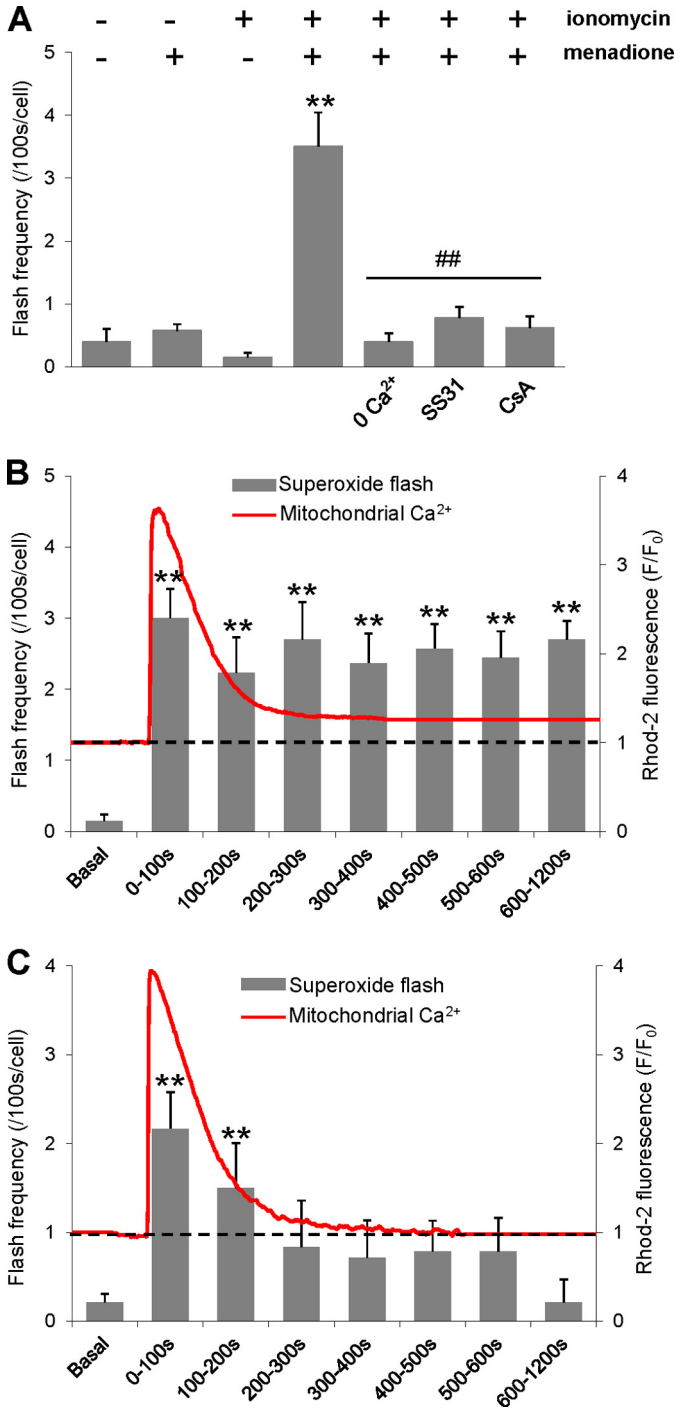

FIGURE 5. **Synergistic effect of mitochondrial Ca^{2+} uniport and ROS elevation on superoxide flash activity.** *A*, synergistic triggering of superoxide flashes by subthreshold menadione (25 μM) combined with ionomycin (2 μM). This effect was blocked by extracellular Ca^{2+} removal (EGTA 5 mM) and by treatment with SS31 (100 μM) or cyclosporine A (*CsA*, 2 μM). The data are expressed as the means ± S.E. (n = 10–40 cells/group). **, $p < 0.01$ *versus* control; ##, $p < 0.01$ *versus* menadione-treated group. *B* and *C*, *bar graphs* showing time-dependent superoxide flash activity induced by ionomycin (2 μM, *B*) or histamine (100 μM, *C*) in combination with 25 μM menadione. The *red trace* overlay reflects mitochondrial Ca^{2+} measured by Rhod-2. A *dashed line* marks the basal mitochondrial Ca^{2+} level before ionomycin or histamine stimulation. The data are expressed as the means ± S.E. (n = 10–60 cells/group). **, $p < 0.01$ *versus* control.

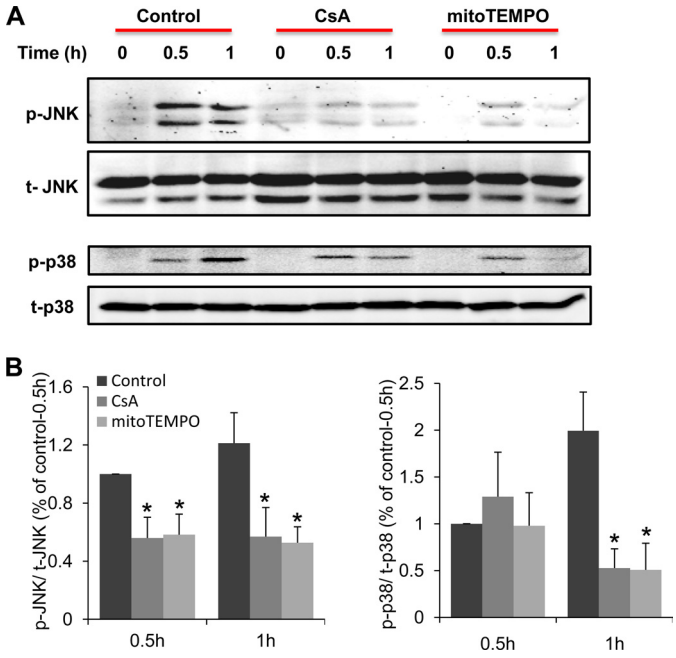

FIGURE 6. **Role of superoxide flashes in hyperosmotic stress-induced activation of JNK and p38.** HeLa cells were pretreated with or without cyclosporine A (*CsA*, 2 μM, 0.5 h) or mitoTEMPO (500 nM, 0.5 h) and then challenged with high glucose for different time (0, 0.5, and 1 h). Cell lysates were subjected to immunoblotting with anti-phospho-JNK (*p-JNK*), total JNK (*t-JNK*), phospho-p38 (*p-p38*), and total p38 (*t-p38*) antibodies. *A*, representative Western blot results. *B*, statistics. The ratios of p-JNK to t-JNK and phospho-JNK to total p38 were normalized with that for 0.5-h high glucose challenge alone. The data are expressed as the means ± S.E. (n = 3 for JNK and n = 4 for p38). *, $p < 0.05$ *versus* control.

flashes with either CsA or mitoTEMPO largely reduced the phosphorylation of JNK and p38 upon high glucose challenge (Fig. 6). This result indicates that the mitochondrial superoxide flashes play important roles in MAPK activation.

DISCUSSION

Mitochondrial Ca^{2+}- and ROS-induced Activation of Superoxide Flashes—The present study has demonstrated that both the Ca^{2+}-induced flash activation (CIFA) mechanism and the ROS-induced flash activation (RIFA) mechanism regulate the stochastic gating of the mPTP and the resultant quantal ROS production in living cells at the single-mitochondrion level. Specifically, hyperactivity of mitochondrial superoxide flashes during hyperosmotic stress depends on the mitochondrial Ca^{2+} uniport and elevated basal ROS production. Removal of extracellular Ca^{2+}, knockdown of the critical mitochondrial uniporter components MICU1 and MCU, or scavenging ROS with mitochondria-targeted antioxidants all effectively diminished the flash response to hyperosmotic stress. Moreover, directly elevating mitochondrial basal ROS production with menadione dose-dependently increased the flash activity. Thus, the demonstration of CIFA and RIFA mechanisms underscores the interconnectedness between superoxide flashes and other processes vital to mitochondrial function.

More importantly, we have demonstrated a powerful Ca^{2+}- and ROS-induced flash activation (CRIFA) mechanism, *i.e.*, a profound synergism between concurrent CIFA and RIFA in triggering superoxide flashes. In contrast to the prominent mitochondrial Ca^{2+}-dependent component of superoxide flash

activity (defined as the portion of flash activity that was inhibited by MCU or MICU1 knockdown) in hyperosmotic stress, CIFA acting alone was unable or inefficacious in triggering superoxide flashes such that mitochondrial Ca^{2+} transients during histamine stimulation or those induced by ionomycin at 2 μM elicited no appreciable increase in flash activity. The poor efficiency of CIFA is consistent with the apparent lack of effect of Ca^{2+} transients on the flash activity in beating cardiac myocytes (17) and the small effect of tetanic stimulation-elicited Ca^{2+} elevation on the flash production in skeletal muscle (22). Likewise, RIFA acting alone was also relatively inefficient in triggering flash activity compared with the ROS-dependent component of the flash response to hyperosmotic stress (defined as the portion that was inhibited by a mitochondria-targeted antioxidant), where both CIFA and RIFA were in action. Menadione at 25 μM, while enhancing mitochondrial ROS production to a level similar to that in hyperosmotic stress, was subthreshold in terms of flash activation; it required several-fold more intense ROS production to elicit a flash response comparable with the hyperosmotic flash response. Strikingly, when a poor flash-activating Ca^{2+} signal was combined with a subthreshold ROS signal, they together induced intense flash activity with the rate of flash occurrence reaching 3.5 events/100 s/cell, attesting to the power of CRIFA. These results strongly support a specific role of superoxide flashes as a coincidence detector of mitochondrial Ca^{2+} and ROS in physiological and pathophysiological circumstances.

In physiological settings, CRIFA may operate with substrate-stimulated respiration because superoxide flash activity is increased with elevated mitochondrial biogenetic status in skeletal muscle cells (19, 21, 22). In pathophysiological settings, immediately following reoxygenation after hypoxia or anoxia, when mitochondrial Ca^{2+} overload and oxidative stress are expected to occur concurrently, we have also found intense superoxide flash activity (17, 27) that may reflect CRIFA at work. Furthermore, that hyperosmotic stress potently augments superoxide flash activity can be explained because it simultaneously elevates mitochondrial Ca^{2+} and ROS signals that act in synergy. As demonstrated here, controlling the synergy by manipulating the mitochondrial Ca^{2+} uniport or targeting mitochondrial ROS or both affords effective means to modulate the flash response mediated by the CRIFA mechanism.

It is noteworthy that high glucose and high mannitol elicited differential flash responses largely in accordance with their respective Ca^{2+} responses. As compared with equal molar high mannitol, high glucose stimulated ~2-fold greater mitochondrial Ca^{2+} uniport and 1.8-fold greater superoxide flash activity. This difference shows that high glucose does not elicit a simple hyperosmotic stress. As an important metabolite in addition to being an osmolyte, glucose may stimulate metabolic signaling at the plasma membrane, cytosolic, and organelle levels, which will further modulate mitochondrial Ca^{2+} and flash responses.

Novel Properties of the mPTP Underlying the Flash—For pMPT mediated by irreversible mPTP opening, it has been well established that mitochondrial Ca^{2+} overload is a robust activator and that ROS potentiates the Ca^{2+} effect (48). Both the irreversible and the superoxide flash-coupled mPTP openings are associated with abrupt dissipation of $\Delta\Psi_m$, mitochondrial swelling, and the nonselective passage of small solutes between matrix and cytosol. Moreover, superoxide flashes, except for those in skeletal muscle, are CsA-sensitive, as is the case with the pMPT. The present finding further extends the similarities between tMPT and pMPT in terms of synergistic regulation by mitochondrial Ca^{2+} and ROS signals.

Equally important, the present study revealed a remarkable difference between the Ca^{2+}-dependent activity of the two types of mPTP activity. Although irreversible opening of the mPTP usually requires pathological overloading of mitochondrial Ca^{2+} to the range of 10–40 μM (49, 50), such overload is not a prerequisite for the transient opening of the flash-coupled mPTP. The mitochondrial Ca^{2+} levels in all the present experiments can be categorized as physiological: because time course plots revealed no sign of saturation of the mitochondrial Ca^{2+} indicator Rhod-2 (K_d = 570 nM), even the peak mitochondrial Ca^{2+} (Rhod-2 $\Delta F/F_0$ ~3) involved should be no more than a few micromoles. Strikingly, sustained hyperactivity of superoxide flashes was supported by an even lower steady-state mitochondrial Ca^{2+} that usually corresponded to a $\Delta F/F_0$ of 0.3–0.8. These results provide strong evidence that physiological elevation of mitochondrial Ca^{2+} as small as a fractional increase of the resting level can greatly potentiate the transient opening of the mPTP in intact cells, if there is a concurrently elevated ROS production.

The irreversible pMPT is a threshold-dependent phenomenon and displays irreversible kinetics and a high Ca^{2+} dependence (11, 51). However, the superoxide flash-coupled tMPT activity is regulated over a broad dynamic range in a graded fashion, *e.g.*, by manipulating the mitochondrial Ca^{2+} uniport, ROS production, or ROS clearance. Taken together, we prefer a parsimonious model in which the flash-coupled tMPT and the irreversible pMPT stem from the same mPTP in terms of the core elements, but operating in entirely different regimes or with distinct ancillary modifications. Nevertheless, an alternative possibility is that they originate from distinctive large conductance permeability channels, but with overlapping regulatory mechanisms.

Possible Role of Superoxide Flashes in Hyperosmotic Stress—Hyperosmotic stress occurs in pathological states such as ischemia, septic shock, and diabetic coma (52, 53). Mitochondria play important roles in protecting the cell against such stress (29, 54). In the present study, we have demonstrated that superoxide flash activity is markedly and reversibly increased in response to hyperosmotic stress, indicating that superoxide flash production, as well as tMPT, is a novel and prominent component of the osmotic stress response.

Among all known flash regulators, hyperosmotic stress appears to be stronger than insulin- or substrate-induced metabolic signaling in skeletal muscle cells (21) or apoptotic oxidative stress induced by selenite or hydrogen peroxide in HeLa cells (18). Unabated in the presence of high osmolytes, hyperosmotic stress-induced flash response is also more persistent than the bursting superoxide flash hyperactivity in response to reoxygenation after hypoxia or anoxia in cardiomyocytes (17, 27). Functionally, stochastic, transient mPTP opening may play

a role in mitochondrial functional regulation, cellular signaling, and stress responses. Indeed, superoxide flashes in response to hyperosmotic stress participate in activating JNK and p38, which are essential for inducing cellular adaptive responses. Unlike pMPT, tMPT in a superoxide flash is not associated with the release of proapoptotic molecules (18) and is completely reversible. In particular, it has been postulated that tMPT plays a role in mitochondrial Ca^{2+} homeostasis by affording a Ca^{2+}-induced Ca^{2+} release mechanism (14) or reset metabolic status (17). Thus, the flash genesis, which is potently regulated by the CRIFA mechanism, is well positioned as the early cellular stress response to invoke both cell-protective and preconditioning actions.

In summary, superoxide flashes are dually regulated by mitochondrial Ca^{2+} (via CIFA) and ROS (via RIFA) and are potently activated by concurrent mitochondrial Ca^{2+} and ROS signals (via CRIFA). Differing from synergistic Ca^{2+} and ROS regulation of the pMPT in injurious or extremely stressful conditions, the CRIFA mechanism operates in the physiological Ca^{2+} regime and plays an important role in the cellular response to mild to moderate stress. The similarities and disparities between regulation of the flash genesis and the pMPT shed new light on the nature of the mPTP underlying the flash and underscore the complexity of cellular ROS signaling.

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